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Rapid activation by fungal elicitor of genes encoding "pathogenesis-related" proteins in cultured parsley cells

(two-dimensional gels/cloned cDNAs/RNA blot hybridization/nuclear run-off transcription)

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ABSTRACT **Administration of a cell-wall preparation from the fungus *Phytophthora megasperma* f. sp. *glycinea*, which acts as an elicitor of phytoalexin production in cell suspension cultures of parsley (*Petroselinum crispum*), also results in a rapid and dramatic increase in the relative amounts of mRNAs coding for a number of small proteins having low isoelectric points. According to various operational criteria, the translation products are classified as "pathogenesis-related" (PR) proteins. Here we report that the cDNA inserts of two pBR322-derived plasmids, pcPR1 and pcPR2, are homologous to mRNAs coding for one (PR1) and three (PR2) of these proteins in hybrid-selected *in vitro* translation experiments. Nuclear run-off transcription studies show that activation of the corresponding genes is extremely rapid; we observed a 4-fold increase in the transcription rate of the PR1 gene within 5 min and a 3-fold increase for the PR2 gene within 20 min following elicitation. Subsequent increases in the amounts of PR1 and PR2 mRNAs indicate that regulation of PR protein synthesis occurs at the transcriptional level.**

Infection of plants with pathogens results in the induction of numerous host-specific biochemical responses, some of which are critical for the ability of the plant to withstand diseases. Although direct evidence is still lacking, a number of mechanisms, including the accumulation of antimicrobial phytoalexins, the enhancement of the activity of certain hydrolytic enzymes, and the *de novo* synthesis of proteins termed "pathogenesis-related" (PR) proteins, appear to play a role in the induced defense response of various plant species (1, 2).

PR proteins were first detected in tobacco cultivars reacting hypersensitively to tobacco mosaic virus (3) but have now been found in various plant species on infection with viruses, viroids, fungi, and bacteria as well as during specific developmental stages of plants (4). These proteins have in common low molecular weights, extreme isoelectric points, and high resistance to both endogenous plant proteinases and commercial preparations such as trypsin, proteinase K, etc. (4). Little, however, is known concerning their cellular localization and function. Their classification is purely operational and may require more precise definition once functions can be assigned to the proteins.

Many active defense reactions are dependent on host protein and RNA synthesis (1, 2). It has been shown previously that parsley cell cultures treated with a cell-wall preparation derived from a soybean pathogen, the fungus *Phytophthora megasperma* f. sp. *glycinea*, produce and accumulate furanocoumarins, their putative phytoalexins (5). More recently, it has been shown that the increase in activity of two enzymes of general phenylpropanoid metabolism involved in furanocoumarin accumulation, phenylalanine ammonia-lyase (EC 4.3.1.5) and 4-coumarate:CoA ligase (EC 6.2.1.12), is due to *de novo* mRNA and protein synthesis and is controlled at the transcriptional level (6, 7).

Here we report the rapid synthesis, following elicitor treatment, of a number of low molecular weight proteins having low isoelectric points that distinguish them as PR proteins of parsley. Using two cDNA clones, we found a rapid and large accumulation of the mRNAs in the cells. Furthermore, nuclear run-off experiments show that control is mainly at the level of transcription and that the induction of the respective genes occurs within minutes following addition of the elicitor to the cell cultures.

MATERIALS AND METHODS

Cell Cultures. Cell suspension cultures of parsley (*Petroselinum crispum*) were grown in the dark as described (8). They were used approximately 7 days after subculturing.

Plasmids/cDNA Inserts. The origin and preparation of the cDNAs by reverse transcription of poly(A)$^+$ RNA along with identification of the pcPAL, pc4CL, and pcCHS clones have been reported previously (6, 9). The pcLF14 clone represents a constitutively expressed parsley gene of unknown function (7). Identification of the pcPR1 and pcPR2 clones was achieved by hybrid-selection translation (see below). Plasmids were isolated using the method of McMasters *et al.* (10). Insert isolation from the plasmids was by *Pst* I digestion followed by electroelution from agarose gels into dialysis bags. The cDNA inserts had the following sizes, in base pairs: 1700, cPAL; 1500, cCHS; 2400, cLF14; 850, cPR2; and 380, cPR1. Because of alteration of one *Pst* I site during cloning, isolation of the c4CL insert from the plasmid was not possible. For these experiments we used the *Pst* I/*Eco*RI fragment of the plasmid, which contained the 450-base-pair insert along with 750 base pairs of pBR322. In some experiments *Pst* I-linearized pBR322 was used as a control for unspecified binding to the filters.

Elicitor Treatment. Heat-released elicitor from the fungus *Ph. megasperma* f. sp. *glycinea* was prepared by the method of Ayers *et al.* (11). Cultures were treated with 30–60 μg (dry weight) of elicitor per ml of medium and further incubated in the dark at 26°C with constant shaking.

RNA Isolation. Total RNA for RNA blot hybridization and *in vitro* translation was isolated according to Kuhn *et al.* (6). Ethanol-precipitated RNA was washed with 3 M sodium acetate and then reprecipitated.

RNA Blot Hybridization. Total RNA (20 μg) was denatured and separated on formaldehyde/agarose gels. Conditions of denaturing, electrophoresis, and the capillary blotting procedure were as described (6). Blotted RNA was hybridized to the various nick-translated ^{32}P-labeled cDNAs (12) in 50% formamide/10% dextran sulfate/1 M NaCl/10× Denhardt's solution (0.2% bovine serum albumin/0.2% Ficoll 400/0.2%

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Abbreviations: PR, pathogenesis related; PR1 and PR2, PR proteins 1 and 2.

polyvinyl pyrrolidone)/0.2% sodium pyrophosphate/1% $NaDodSO_4$/50 mM Tris·HCl, pH 7.5, containing denatured DNA (salmon testis) at 100 μg/ml and ^{32}P-labeled cDNA probe at 10 ng/ml at 42°C for 24 hr. Blots were washed and autoradiographed at −70°C using Trimax (3 M) film and an intensifying screen. Autoradiograms were scanned with an LKB laser densitometer.

For RNA dot-blot experiments, the conditions described above were used except that the RNA (1 μg per dot) was applied directly onto GeneScreen filters using a Hybridot Manifold (Bethesda Research Laboratories).

Labeling of Proteins *in Vivo*. Cultured parsley cells were treated with elicitor for 6 hr. One hour before harvesting, treated and untreated cells were labeled by adding 10 μCi of [^{35}S]methionine (1 Ci = 37 GBq) per ml of medium. Cells were extracted and equal amounts of radioactivity were separated on two-dimensional gels.

Two-Dimensional Gel Electrophoresis. Two-dimensional polyacrylamide gel electrophoresis was carried out essentially according to the method of O'Farrell (13). The first-dimension gel contained a 1:2:2 mixture of pH 3.5–10, pH 4–6, and pH 6–8 ampholines. The second dimension was a 10–18% polyacrylamide gradient. Fluorography was as described by Bonner and Laskey (14).

Hybrid-Selection Translation. Plasmid DNAs (2 μg per filter) were denatured and immobilized on 1-cm^2 nitrocellulose filters, which were then rinsed with 1.5 M NaCl/150 mM trisodium citrate, air dried, and baked for 1 hr at 80°C. DNAs were hybridized to 200 μg of total RNA in 50 mM Pipes, pH 7.5/1 M NaCl/1% $NaDodSO_4$/30% formamide for 15–24 hr at 47°C. Filters were washed as described above for RNA blot hybridization. RNA was released by boiling filters in water, coprecipitated with 20 μg of yeast tRNA, and translated *in vitro* using a reticulocyte lysate (Amersham).

Nuclei Isolation/*in Vitro* Transcription. The isolation of transcriptionally active nuclei was achieved by using a modified (7) method of Willmitzer and Wagner (15). Run-off transcription was performed in 100- or 200-μl reaction volumes using 100 (200) μg of DNA and 100 (200) μCi of [^{32}P]UTP (410 Ci/mmol; Amersham) as recently reported (7). The *in vitro*-synthesized labeled RNA was extracted according to the method of McKnight and Palmiter (16).

DNA Blot/Dot-Blot Hybridization. For blot hybridization of *in vitro*-labeled RNA transcripts, 200 ng of each cDNA insert was electrophoresed on 1.2% agarose gels and transferred to GeneScreen*Plus* filters. In dot-blot experiments, the inserts were dotted directly onto the filters by using a Hybridot Manifold (Bethesda Research Laboratories). Prehybridization treatment of the filters, conditions for hybridization with [^{32}P]RNA, and the filter-washing procedure were as described previously (7).

RESULTS

Two-Dimensional Gel Analysis. The pattern of *in vivo*-labeled proteins extracted from elicitor-treated cells differed significantly from that of untreated control cells when analyzed on two-dimensional polyacrylamide gels (J.B. and K.H., unpublished work). One of the most obvious differences was the appearance in elicitor-treated cells of several spots corresponding to low molecular weight proteins having isoelectric points ranging from 4.4 to 4.7 (Fig. 1, boxed area). The molecular weights of these proteins ranged from 17,500 to 19,200. The appearance of these small acidic proteins, operationally defined as PR proteins (3), was detected 2 hr after administration of elicitor to the cells.

Translation of Hybrid-Selected mRNA. The PR proteins could be detected equally well on two-dimensional gels following *in vitro* translation of total RNA extracted from elicitor-treated cells (Fig. 2*A*). The use of two different

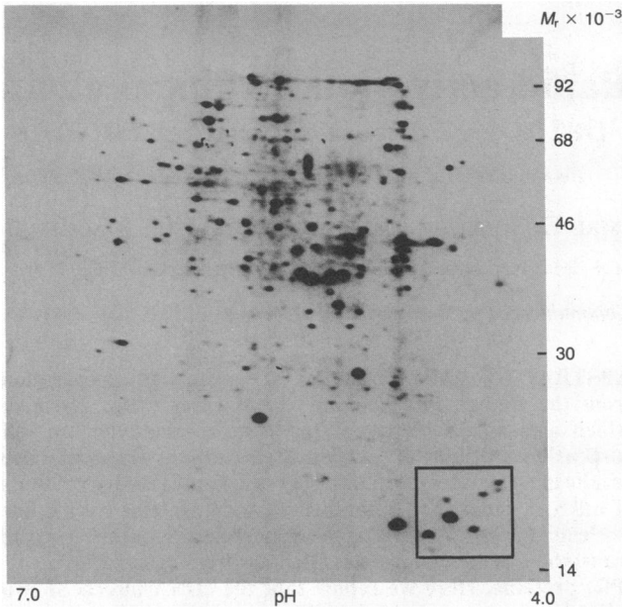

FIG. 1. Fluorograph of a two-dimensional polyacrylamide gel of proteins synthesized *in vivo* by cultured parsley cells. The cells were treated with elicitor 6 hr prior to harvest and labeled with [^{35}S]methionine during the last 60 min of incubation with elicitor. The boxed area marks the induced PR proteins (this area is shown in Fig. 2). Molecular weight markers were as follows: lysozyme (14,300), carbonic anhydrase (30,000), ovalbumin (46,000), bovine serum albumin (68,000), and phosphorylase b (92,500).

parsley cDNA clones, pcPR1 and pcPR2, in hybrid-selection translation experiments with total RNA from stimulated cells resulted in the selection of mRNAs coding for four PR proteins. Although pcPR1 selected for an RNA whose translation yielded one low molecular weight protein, the other DNA probe, pcPR2, gave rise to the formation of three distinct polypeptides.

To relate the hybrid-selection translated products to those observed in the labeling experiments with total RNA, we analyzed appropriate mixtures on two-dimensional polyacrylamide gels. Addition of the pcPR1-selection-translated product to the total translation products markedly increased the radioactivity of one spot (Fig. 2*B*). The polypeptide had a molecular weight of about 17,600 and an isoelectric point of 4.7. Addition of the pcPR2-selection-translated products resulted in increased radioactivity of three spots (Fig. 2*C*). These three polypeptides appear to be the largest ($M_r \approx$ 19,000) and the most acidic (pI 4.4, 4.5) of this group of

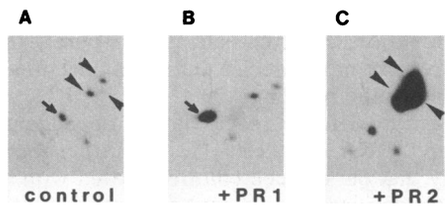

FIG. 2. Identification of the PR1 and PR2 proteins. Small sections of two-dimensional gels are shown as indicated by the box in Fig. 1. RNA was hybrid-selected with the PR1 or the PR2 cDNA insert and translated *in vitro*. The products were mixed with the labeled proteins obtained by *in vitro* translation of total RNA isolated 6 hr after treatment of cells with elicitor. (*A*) Products of total RNA translation. (*B* and *C*) Mixtures of total RNA translation products (cf. *A*) with the PR1 and PR2 hybrid-selection-translation products, respectively. Arrows, PR1 proteins (*A* and *B*); arrowheads, PR2 proteins (*A* and *C*).

proteins in parsley cells. They clearly differed from the one detected by using the pcPR1 probe.

Kinetics of PR1 and PR2 mRNA Induction. From RNA blot hybridization experiments, using total RNA, we estimate the lengths of the PR1 and PR2 mRNAs to be 750–900 and 800–1000 nucleotides, respectively (Fig. 3 *A* and *B*). These experiments also show that the quantity of these mRNAs was about 50 times greater than that of 4-coumarate:CoA ligase (4CL) mRNA, previously reported elicitor-induced gene product (Fig. 3 *C* and *D*). Use of total cellular RNA isolated at various times from elicitor-treated cells and hybridization with the appropriate cDNAs in RNA dot-blot experiments demonstrated the rapid appearance of newly synthesized PR1 and PR2 mRNAs (Fig. 3*E*). An increase was detected within 1 hr of elicitor treatment of the cells. The amount of hybridizable mRNA reached a maximum after 5 hr in both cases and then decreased. In some experiments a second increase was observed at later stages.

***In Vitro* Run-Off Transcription.** To investigate whether the increase in the rate of PR1 and PR2 protein synthesis was the result of increased transcription rates, we performed nuclear run-off transcription experiments *in vitro* with nuclei isolated from cells treated for various lengths of time with elicitor. Since under these conditions reinitiation of transcription was negligible, the amount of [^{32}P]UTP incorporated into a specific RNA transcript directly reflected the amount of RNA polymerase activity associated with the respective gene *in vivo* at the time of nuclei isolation. The labeled RNA was hybridized to immobilized cDNA under conditions of DNA excess. As shown in Fig. 4, the stimulation of *PR1* and *PR2* gene expression was very rapid, reaching a maximum between 0.5 and 1 hr after elicitor treatment and then decreasing for another 2 hr before increasing again. The transcription rates for these genes stayed at high levels for at least 15 hr after exposure of the cells to elicitor. Although the timing and extent of the transient reduction of the rate of transcription observed here between the 1st and 3rd hr varied from experiment to experiment, it was always observed. By scanning various autoradiographs we calculated that elicitor treatment caused an approximately 20-fold (*PR1*) and a 30- to 50-fold (*PR2*) stimulation of transcription at its maximum, relative to the basal expression levels in untreated cells. By comparison, the maximal transcription rates of the elicitor-inducible genes encoding phenylalanine ammonia-lyase and 4-coumarate:CoA ligase (*PAL* and *4CL*, respectively) were a factor of 4–6 lower than the transcription rates observed for the *PR* genes within this period of time. Each strip blot in these experiments also contained two additional cDNA inserts as controls. The insert from pcLF14 represents a gene whose expression has been shown to be constitutive in parsley cells (7). The other insert, obtained from pcCHS, codes for the enzyme chalcone synthase (*CHS*). *CHS* gene expression is activated and regulated by UV light but is not affected by elicitor (7).

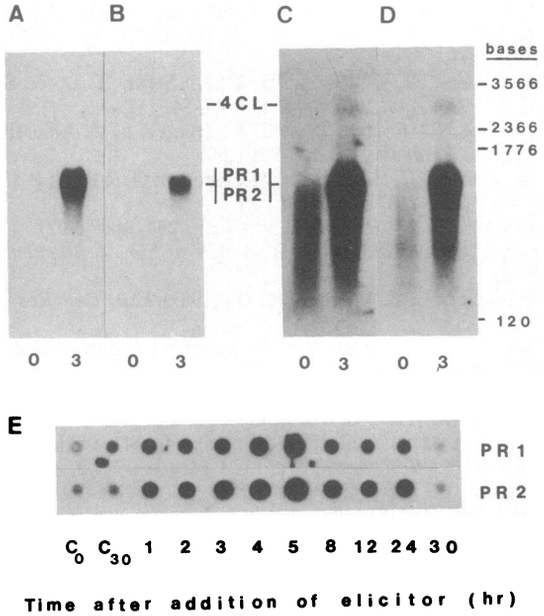

FIG. 3. (*A–D*) Blot hybridization of RNA from untreated (0 hr) and elicitor-stimulated (3 hr) cells. Hybridization probes were the PR1 and PR2 cDNA inserts and the *Eco*RI/*Pst* I fragment of pc4CL. Two exposures of the same gel containing total RNA separated by electrophoresis and hybridized to mixtures of ^{32}P-labeled 4-coumarate:CoA ligase (*4CL*) and *PR1* (*A* and *C*) or *PR2* (*B* and *D*) probes are shown. Size markers (indicated on the right) were *Escherichia coli* and calf thymus rRNAs. (*E*) Dot-blot analysis of the time course of PR1 and PR2 mRNA induction in elicitor-stimulated cells. RNA from untreated cells at 0-hr (C_0) and 30-hr (C_{30}) time points was used as reference.

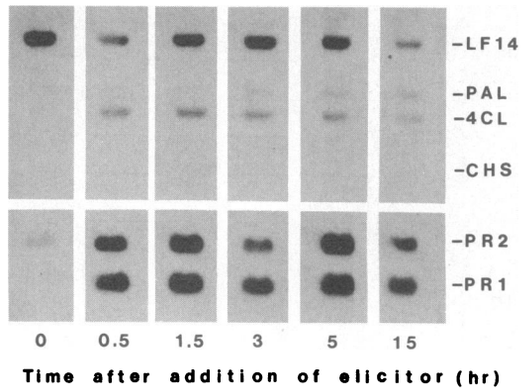

FIG. 4. DNA blot hybridization of *in vitro*-labeled run-off transcribed RNA from nuclei isolated at the indicated times after treatment of cells with elicitor. RNAs having identical amounts of radioactivity were hybridized to filters containing 200 ng each of the individual cDNA probes. *LF14*, *PAL*, *4CL*, and *CHS* (upper filter) and *PR1* and *PR2* (lower filter) cDNA inserts were separated in neighboring lanes on the same gel and blotted, and the filters were hybridized with the labeled RNA.

The rapid stimulation of transcription of the *PR1* and *PR2* genes was further examined in short-term kinetic studies using the DNA dot-blot method to increase the sensitivity of detection (Fig. 5*A*). As the autoradiographs show, an increase in the rate of transcription of the *PR1* gene was detectable in nuclei derived from cells treated for only 5 min with elicitor. An increase in the *PR2* gene was apparent after 20 min of elicitor treatment. By densitometric scanning of the autoradiographs, we estimate a 4-fold increase in transcription rate of the *PR1* gene within 5 min and a 3-fold increase for the *PR2* gene after 20 min of elicitor treatment of the cells (Fig. 5*B*).

Essentially the same results as shown here were obtained in several independent experiments.

DISCUSSION

We have shown that a number of acidic, low molecular weight proteins are rapidly synthesized following elicitor treatment of parsley cell cultures. *De novo* mRNA synthesis is largely responsible for their accumulation, as shown by using plasmids containing two PR-specific cDNAs, pcPR1 and pcPR2, that hybrid-select for mRNAs encoding one and three of these proteins, respectively.

Transcription studies with isolated nuclei from control and elicitor-treated cells indicate that these *PR* genes are regulated mainly at the transcriptional level. Stimulation of

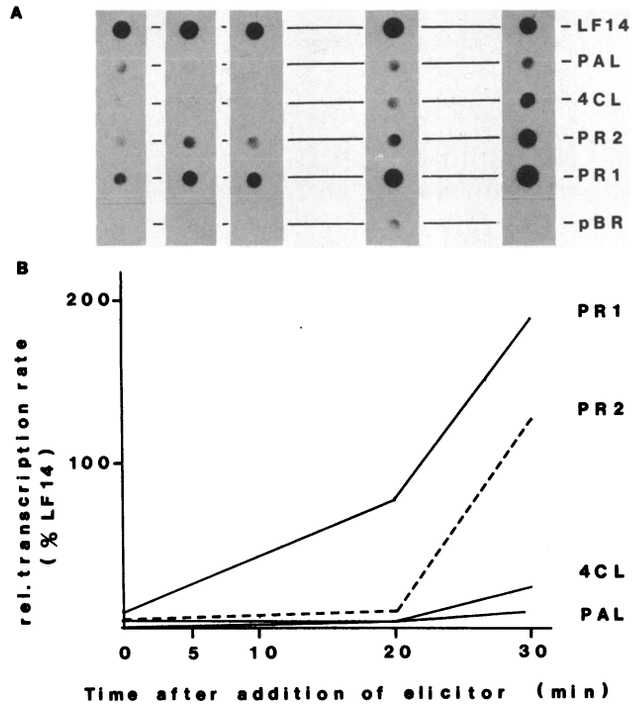

FIG. 5. Short-term kinetics of changes in *PR1*, *PR2*, *PAL*, and *4CL* transcription rates after treatment of parsley cells with elicitor. Nuclei were isolated at the times indicated. Labeled RNA run-off transcripts were hybridized to cDNAs that had been dotted on GeneScreen*Plus* filters. The radioactivity was visualized by autoradiography (*A*) and relative signal intensities were calculated by densitometric scanning of the autoradiographs (*B*) using the LF14 signal as an internal standard.

transcription was extremely rapid and occurred within minutes after elicitation. Despite the great similarity of the overall kinetics of the changes in the *PR1* and *PR2* transcription rates, it remains open at this stage whether or not the two genes are coordinately regulated.

So far as we know, these are the earliest transcriptional events reported for specific genes in exogenously stimulated plant cells. The induction kinetics are comparable with those found in various mammalian systems following stimulation with interferon, estradiol, or thyroid hormones (17–19).

The products encoded by the *PR1* and *PR2* genes are similar in various respects to a class of proteins termed PR proteins in other plant species after infection with various pathogens or other causes of pathogenesis—e.g., treatment with certain chemicals (4). In contrast to the transcriptional regulation of *PR* genes in parsley cells described here, regulation of PR-protein accumulation in infected tobacco plants is reported to be at the translational level (20). Further studies will have to clarify whether different regulatory mechanisms are involved in *PR* gene expression in different plants.

As shown by hybrid-selection translation, the PR2 cDNA selects for mRNAs encoding three different PR proteins. This leads to the speculation that the PR2 proteins are products of a small gene family.

Although the role of PR proteins in disease resistance is not known and we have greatly reduced the natural complexity of the plant–pathogen interaction by treating cultured cells with a pathogen-derived elicitor, the rapid and intense activation of *PR* gene transcription as well as the subsequent large accumulation of the respective mRNAs are intriguing. Resistant and susceptible cultivars of a given plant often show the same biochemical defense reactions when challenged by a pathogen, but differ in the speed and intensity of the response (1, 21). Thus, genes with the ability to respond so strongly and rapidly as the *PR* genes are of great interest for studies of the molecular mechanisms of disease resistance in plants. Such reactions are expected to occur in the localized, hypersensitive response of plants in incompatible interactions with pathogens, particularly when a nonhost plant (e.g., parsley) defends itself very efficiently against invasion by a pathogen of another species (e.g., the soybean pathogen *Ph. megasperma* f. sp. *glycinea*) (22). For the same reason, the *PR* genes should be attractive for studies of the mechanisms involved in the regulation of gene expression in plants.

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